



# Parkinson's Disease Is Associated with Oxidative Damage to Cytoplasmic DNA and RNA in Substantia Nigra Neurons

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Oxidative damage, including modification of nucleic acids, may contribute to dopaminergic neurodegeneration in the substantia nigra (SN) of patients with Parkinson's disease (PD). To investigate the extent and distribution of nucleic acid oxidative damage in these vulnerable dopaminergic neurons, we immunohistochemically characterized a common product of nucleic acid oxidation, 8-hydroxyguanosine (8OHG). In PD patients, cytoplasmic 8OHG immunoreactivity was intense in neurons of the SN, and present to a lesser extent in neurons of the nucleus raphe dorsalis and oculomotor nucleus, and occasionally in glia. The proportion of 8OHG immunoreactive SN neurons was significantly greater in PD patients compared to age-matched controls. Midbrain sections from patients with multiple system atrophy-Parkinsonian type (MSA-P) and dementia with Lewy bodies (DLB) also were examined. These showed increased cytoplasmic 8OHG immunoreactivity in SN neurons in both MSA-P and DLB compared to controls; however, the proportion of positive neurons was significantly less than in PD patients. The regional distribution of 8OHG immunoreactive neurons within the SN corresponded to the distribution of neurodegeneration for these three diseases. Nuclear 8OHG immunoreactivity was not observed in any individual. The type of cytoplasmic nucleic acid responsible for 8OHG immunoreactivity was analyzed by preincubating midbrain sections from PD patients with RNase, DNase, or both enzymes. 8OHG immunoreactivity was substantially diminished by either RNase or DNase, and completely ablated by both enzymes. These results suggest that oxidative damage to cytoplasmic nucleic acid is selectively increased in midbrain, especially the SN, of PD patients and much less so in MSA-P and DLB patients. Moreover, oxidative damage to nucleic acid is largely restricted to cytoplasm with both RNA and

mitochondrial DNA as targets. (*Am J Pathol* 1999, 154:1423-1429)

Parkinson's disease (PD) is characterized clinically by bradykinesia, resting tremor, and rigidity and pathologically by progressive degeneration of dopaminergic neurons in the zona compacta of the substantia nigra (SN).<sup>1</sup> The causes of dopaminergic neurodegeneration in PD remain unclear, but several lines of evidence suggest involvement of oxidative stress in PD pathogenesis. First, PD is associated with both increased levels of nigral iron,<sup>2</sup> a catalytic agent for production of hydroxyl radical ( $\cdot\text{OH}$ ), and increased Mn superoxide dismutase activity.<sup>3</sup> Second, midbrain levels of reduced glutathione are diminished in PD patients.<sup>4</sup> Third, there is evidence of increased oxidative damage in midbrain from PD patients, including lipid peroxidation,<sup>5</sup> protein oxidation,<sup>6</sup> and oxidation of DNA.<sup>7</sup> Finally, several laboratories have observed increased catechol oxidation in the midbrain of PD patients.<sup>8</sup> Catechol oxidation may render dopaminergic neurons especially vulnerable to oxidative stress because metabolism of dopamine and other endogenous catechols produces electrophilic semiquinones and quinones in addition to reactive oxygen species (ROS), eg, superoxide anion ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and  $\cdot\text{OH}$ .<sup>9,10</sup>

There are several deleterious outcomes from excess oxidative stress in nigral neurons. These include modification of macromolecules by ROS or oxidized catechols, depletion of intracellular thiols, and inhibition of mitochondrial function.<sup>9-15</sup> Indeed, some of these processes already have been shown to readily induce cell death in experimental systems.<sup>11,16,17</sup> Among these potential mechanisms of neurodegeneration, oxidation of nucleic acid may be especially damaging because it can result in permanent modifications that may contribute to neurodegeneration over years. This may be particularly true for

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mitochondrial DNA whose repair systems are much less efficient than those of nuclear DNA.<sup>18</sup> Indeed, increasing evidence is now beginning to suggest that some neurodegenerative diseases may derive from defects in mitochondrial DNA.<sup>19</sup>

Mitochondrial point mutations and a 4977-bp "common deletion," both of which are likely related to oxygen radical attack, have been reported in some studies to occur either exclusively or with increased frequency in midbrain from patients with PD.<sup>19-22</sup> However, subsequent studies could not confirm these results; rather, it was suggested that the mitochondrial point mutations and common deletion are associated with aging and not PD.<sup>23,24</sup> One possibility for these apparently conflicting results is the inherent problem in trying to quantify mitochondrial DNA mutations in whole midbrain while only a subset of midbrain neurons are degenerating in PD. Using a different experimental approach, others have measured 8-hydroxyguanine, a product of free radical attack, in SN of PD patients and controls using gas chromatography with mass spectrometric detection.<sup>7</sup> These investigators showed that 8-hydroxyguanine levels were increased in PD patients, although it is not known how much it is derived from oxidative damage to mitochondrial versus nuclear DNA. Importantly, all of these techniques are limited because they have not addressed either the cell types or the subcellular distribution of these DNA mutations or adducts within midbrain from PD patients.

In this study, we have tested the hypothesis that oxidative damage to nucleic acid in midbrain of PD patients is present largely in the nigral neurons, possibly within mitochondria, using an immunohistochemical assay for an antibody to one of the common products from nucleoside oxidation, 8-hydroxyguanosine (8OHG). We first determined whether there was any difference in midbrain 8OHG immunoreactivity between age-matched controls and patients with PD. Then we investigated whether 8OHG immunoreactivity was simply a reflection of neurodegeneration or specifically related to PD by quantifying 8OHG staining in patients with multiple system atrophy-Parkinsonian type (MSA-P) or dementia with Lewy bodies (DLB). Finally, since 8OHG antibody binds to both -OH modified DNA and RNA, we assessed 8OHG immunoreactivity with or without pretreatment with DNase and RNase. We found that 8OHG was not only selectively increased in midbrain, especially in SN neurons of PD patients, but also that 8OHG immunoreactivity was limited to the cytoplasm of all cell populations. Moreover, 8OHG signals were present in cytoplasmic RNA in addition to the cytoplasmic DNA, ie, mitochondrial DNA.

## Materials and Methods

### Patients

Brain tissue was obtained from autopsies performed at Vanderbilt University Medical Center or the University of Kentucky Medical Center. All control individuals were volunteers in a rapid autopsy program who had annual

physical and neurological examinations that were within normal limits. Neuropathological examination of control individuals showed age-related changes only. All patients had been diagnosed during life with an extrapyramidal movement disorder or dementia. Final diagnoses were established by neuropathological examination according to established criteria.<sup>1</sup>

### Antibodies

Mouse monoclonal anti-8OHG was kindly supplied by Dr. Regina Santella<sup>25</sup> and the specificity of this antibody in paraffin-embedded tissue has been tested previously and the results show that it binds to both 8OHG and 8OHdG and co-localizes with nucleic acids.<sup>26</sup>

### Immunohistochemistry

Eight- $\mu$ m sections were cut from formalin-fixed, paraffin-embedded blocks. The sections examined included midbrain from patients with PD, MSA-P, or DLB at the level containing the red nucleus and the proximal portion of cranial nerve III. In cases of PD, additional sections were cut from blocks of the cerebellum and cerebral cortex. In cases of DLB, additional sections were cut from blocks of the hippocampus. Tissue sections from the corresponding brain regions were obtained from controls. All sections were hydrated through graded ethanol following deparaffinization with xylene, and then processed on a Ventana ES automated immunohistochemistry system according to the manufacturer's specifications including Ventana's protease I pretreatment for 4 minutes and a standard alkaline phosphatase method with fast red chromogen. Negative controls omitted the primary antibody. Specificity of anti-8OHG was confirmed by absorption of the antibody with purified 8OHG (see Figure 3A).

Following the protease I treatment, additional midbrain sections from four PD patients were treated with RNase-free DNase I and S1 DNase (10 U/ $\mu$ l of each, phosphate-buffered saline, 1 hour at 37°C; Boehringer Mannheim); DNase-free RNase (5  $\mu$ g/ $\mu$ l, phosphate-buffered saline, 1 hour at 37°C, Boehringer Mannheim); a combination of all of these nucleases; or phosphate-buffered saline alone (1 hour at 37°C) before incubation with 8OHG antibody.

### Quantification

The SN at the level of cranial nerve III exit was divided into six anatomical regions based on the method of Fearnley and Lees<sup>27</sup>: ventromedial, ventral intermediate, ventrolateral, dorsomedial, dorsolateral, and pars lateralis regions. Cases containing at least 100 remaining neurons in the SN were used for quantification of total neurons and 8OHG-positive neurons to assure accuracy. Midbrain sections from eight control individuals, six PD patients, four MSA-P patients, and four DLB patients met this criterion. Neuron counting was performed by two independent observers, and the average for each region was used for statistical analysis.

**Table 1.** Clinical Information on Patients Chosen for Study

	Control	PD	MSA-P	DLB
Number	8	6	4	4
Age (years)	76 ± 3	78 ± 5	64 ± 8	75 ± 4
Male/female	3/5	3/3	3/1	2/2
Postmortem interval (h)	3.3 ± 2.1	8.8 ± 4.2	8.0 ± 2.2	4.4 ± 3.2

Age and postmortem intervals are expressed as means ± SEM. One-way analyses of variance for age, gender ratio, and postmortem interval were not significantly different when the four groups were compared ( $P > 0.05$ ).

## Results

### Patient Data

A total of 22 individuals were included in this study. There was no significant difference in age, gender ratio, or postmortem interval among the four groups (Table 1).

### Regional Neuronal Loss

The number of SN neurons in midbrain tissue sections from control individuals was  $252 \pm 4$ ,  $77 \pm 2$ ,  $228 \pm 7$ ,  $62 \pm 2$ ,  $93 \pm 6$ , and  $59 \pm 3$  (mean ± SEM) for ventromedial, ventral intermediate, ventrolateral, dorsomedial, dorsolateral, and pars lateralis regions, respectively. There was significant reduction in the number of SN neurons in PD, MSA-P, and DLB patients compared to age-matched controls. Moreover, nigral neuron loss varied among regions in PD, MSA-P, and DLB patients (Table 2). In cases of PD, neuron loss was greatest in the ventrolateral region and least in the dorsomedial region consistent with previously reported results.<sup>27</sup> In addition to loss of ventral tier neurons, MSA-P and DLB patients displayed greater neurodegeneration in the pars lateralis of the SN than did PD patients. These data also are consistent with reports from other laboratories.<sup>27</sup>

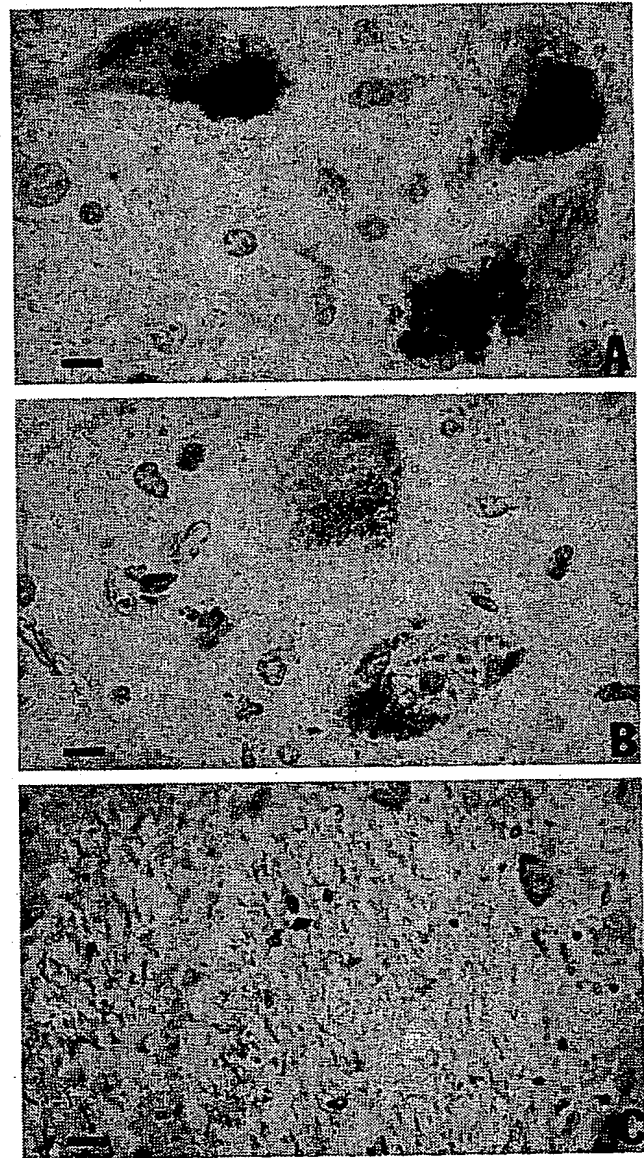
### 8OHG Immunoreactivity

Prominent 8OHG cytoplasmic immunoreactivity was commonly present in soma of SN neurons from patients with PD, MSA-P, or DLB, but was rarely observed in age-matched controls (Figure 1, A and B). There was no correlation between age or postmortem interval and

**Table 2.** Percentage of Remaining Neurons in SN

Region of SN	PD (% control)	MSA-P (% control)	DLB (% control)
Ventromedial	22 ± 1	29 ± 4	17 ± 2
Ventral intermediate	22 ± 2	32 ± 8	16 ± 3
Ventrolateral	9 ± 2	20 ± 4	9 ± 2
Dorsomedial	51 ± 5	58 ± 6	60 ± 5
Dorsolateral	25 ± 3	45 ± 8	30 ± 6
Pars lateralis	33 ± 1	22 ± 6	19 ± 2

Values are remaining neurons in each region of the SN expressed as percentage of controls (means ± SEM). The number of SN neurons in midbrain tissue sections from control individuals was  $252 \pm 4$ ,  $77 \pm 2$ ,  $228 \pm 7$ ,  $62 \pm 2$ ,  $93 \pm 6$ , and  $59 \pm 3$  (mean ± SEM) for ventromedial, ventral intermediate, ventrolateral, dorsomedial, dorsolateral, and pars lateralis regions, respectively.



**Figure 1.** 8OHG immunoreactivity in midbrain structures from patients with Parkinson's disease. 8OHG immunoreactivity is virtually undetectable in age-matched control brain sections (A), while it is abundant in the cytoplasm of SN neurons from PD patients (B). The brown pigments are neuromelanin. In some patients with PD, cytoplasmic 8OHG immunoreactivity was present in midbrain neurons and glia (arrow) outside of the SN (C). Scale bars: A and B, 10  $\mu$ m; C, 25  $\mu$ m.

8OHG formation in any group of patients. Neuronal cytoplasmic 8OHG was granular and did not extend into either dendrites or axon. Nuclear 8OHG immunoreactivity was not observed in any individual. 8OHG immunoreactivity was completely ablated by preabsorbing anti-8OHG with purified 8OHG (Sigma) before incubation with tissue sections (Figure 3A).

Less than 10% of nigral neurons from control individuals were immunoreactive with 8OHG (Table 3 and Figure 2). In these cases, immunoreactive neurons were located primarily in the lateral divisions of the SN and there was no 8OHG immunoreactivity in the midbrain

Table 3. Percentage of 8OHG-Positive Neurons in SN

Region of SN	Control	PD	MSA-P	DLB
Ventromedial	0.0 ± 0.0	57.0 ± 5.0	5.9 ± 1.8	12.1 ± 2.3
Ventral intermediate	0.0 ± 0.0	40.5 ± 5.2	6.9 ± 2.7	14.7 ± 2.8
Ventrolateral	3.8 ± 1.3	59.4 ± 6.1	19.2 ± 5.9	10.1 ± 2.1
Dorsomedial	0.0 ± 0.0	33.1 ± 5.2	7.7 ± 3.9	10.2 ± 2.1
Dorsolateral	0.2 ± 0.1	37.1 ± 5.8	13.7 ± 5.1	16.4 ± 3.3
Pars lateralis	3.8 ± 1.3	53.5 ± 5.2	25.3 ± 4.7	8.3 ± 2.5

Values are 8OHG immunoreactive SN neurons expressed as percentage of total neurons in each region (means ± SEM). One-way analysis of variance comparing each region of the SN among the four groups was statistically significant ( $P < 0.01$ ).

other than these few nigral neurons. The extent and distribution of 8OHG immunoreactive neurons was quite different in PD patients. The remaining nigral neurons of all PD patients showed a significantly greater proportion of neurons immunoreactive for 8OHG compared to controls (Figure 2). In addition, the percentage of 8OHG immunoreactive neurons tended to be greater in the ventral tier than in the dorsal tier, suggesting that the neurons more likely to degenerate also were experiencing more oxidative damage to nucleic acid (Table 3). Lewy bodies were not immunoreactive for 8OHG in any of the patients studied and the intensity of 8OHG immunostaining did not appear to be affected by the presence of Lewy bodies. The percentage of neurons immunoreactive for 8OHG was less in cases of MSA-P and DLB compared to PD despite comparable reduction in the number of SN neurons among these three diseases. One-way analysis of variance comparing the percentage of 8OHG immunoreactive neurons among age-matched controls and patients with PD, MSA-P, and DLB was statistically significant ( $P < 0.01$ ). Repeated paired analyses with Bonferroni correction showed significant differences between controls and PD, MSA-P, or DLB ( $P < 0.05$ ), and between PD and MSA-P or DLB ( $P < 0.05$ ). There was no significant difference in the percentage of 8OHG immunoreactive neurons between MSA-P and DLB.

8OHG immunoreactive cells were present outside of the SN in the midbrain of most PD, some MSA-P and DLB cases, but never in control individuals. The regions of midbrain other than the SN that contained 8OHG immunoreactive neurons were the nucleus raphe dorsalis and oculomotor nucleus. In addition, scattered midbrain glial cells also showed cytoplasmic 8OHG immunoreactivity in some PD, MSA-P, and DLB patients but never in controls (Figure 1C). Overall, the extra-SN midbrain 8OHG immunoreactivity was more extensive in PD than in MSA-P or DLB.

Specificity of cytoplasmic 8OHG immunoreactivity was investigated by examining hippocampal and cerebellar tissue sections from PD and DLB patients and control subjects. Rare pyramidal neuron cytoplasmic 8OHG immunoreactivity was observed in the cerebellum and hippocampus from PD patients and age-matched control subjects (data not shown). In contrast, DLB patients showed substantially more pyramidal neuron cytoplasmic 8OHG immunoreactivity in the hippocampus, corroborating the results of others.<sup>26</sup> Cerebellar 8OHG immunoreactivity for DLB patients was indistinguishable from PD patients and control subjects. Taken together, these findings suggest that neuron cytoplasmic 8OHG immunoreactivity is associated with affected brain regions in these neurodegenerative diseases.

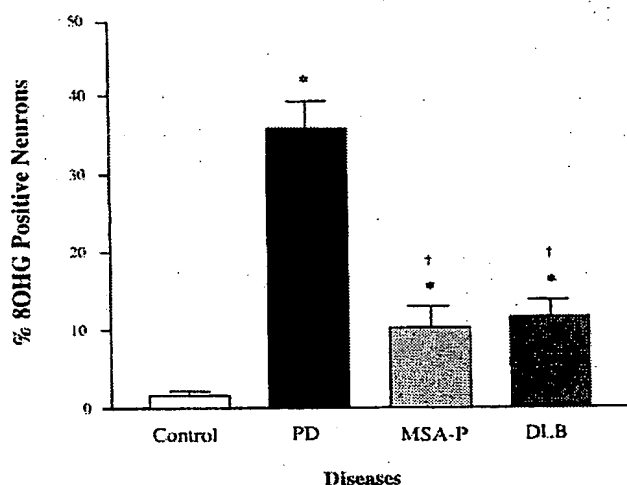
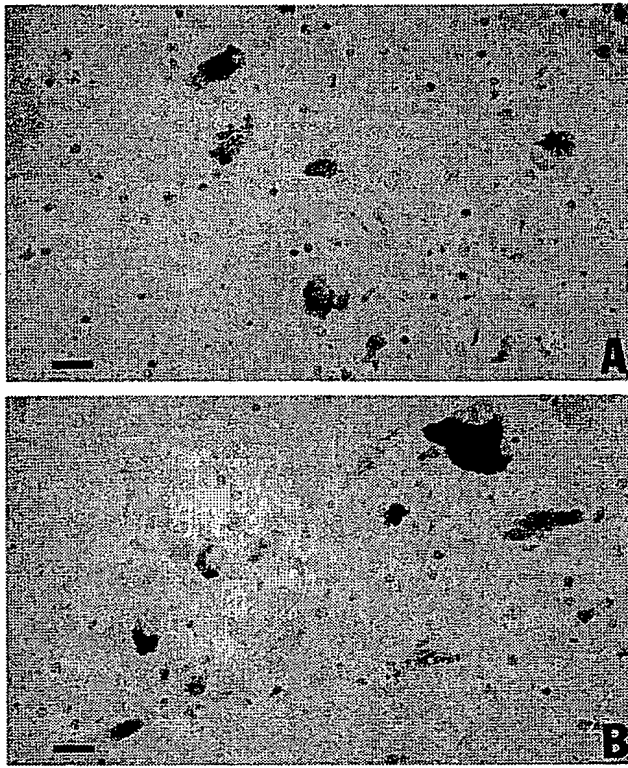


Figure 2. Quantitative Assessment of 8OHG Immunoreactivity. Data are 8OHG immunoreactive SN neurons expressed as percent of total neurons (mean ± SEM). \*:  $P < 0.01$  for control versus PD, MSA-P or DLB. †:  $P < 0.05$  for PD versus MSA-P or DLB.

### Target of Hydroxyl Radical Adduction

Anti-8OHG recognizes ·OH adducts on both DNA and RNA. To analyze the subcellular targets responsible for 8OHG immunoreactivity, immunohistochemical experiments with anti-8OHG were performed with midbrain tissue sections that were preincubated with DNase, RNase, a combination of both enzymes, or buffer alone. DNase or RNase each significantly reduced 8OHG cytoplasmic immunoreactivity, and both enzymes completely eliminated 8OHG immunoreactivity (Figure 3B). These experiments were repeated with DNase or RNase that had been boiled for 30 minutes before incubating with tissue sections. As expected, boiled DNase did not alter 8OHG immunoreactivity while boiled RNase remained active. Protease pretreatment did not decrease 8OHG immunoreactivity as nucleases, a result similar to the data published previously.<sup>26</sup> These data indicate that both mitochondrial DNA and cytoplasmic RNA are targets of oxidative damage to nucleic acid.



**Figure 3.** Effects of Purified 8OHG, DNase and RNase on 8OHG Immunoreactivity. Preabsorption of anti-8OHG with purified 8OHG completely ablated 8OHG immunoreactivity (A). 8OHG immunoreactivity in the SN from PD patients also was completely abolished by pretreatment of tissue with combined DNase and RNase (B). Scale bar = 25  $\mu$ m.

## Discussion

Oxidative damage in the SN has been suggested by many investigators to contribute to selective dopaminergic neurodegeneration in PD. Indeed, several types of oxidative damage have been demonstrated in midbrain tissue from PD patients, including increased levels of iron,<sup>2</sup> decreased levels of reduced glutathione,<sup>2,4</sup> and increased oxidative products of lipid, protein, and DNA.<sup>5-7</sup> It remains unclear whether oxidative damage is restricted to SN neurons in midbrain of PD patients, and what the primary cellular targets of oxidative damage are. The experiments described here were conducted to test the hypothesis that oxidative damage is selectively associated with SN neurons of PD patients by analyzing one of the most common products of oxidative damage to nucleic acid, 8OHG.

Our results demonstrated that severe dopaminergic neurodegeneration was present in the SN of patients with PD, MSA-P or DLB and that the loss of neurons followed the regional distribution described by others.<sup>27</sup> 8OHG immunoreactivity in the remaining SN neurons was increased in PD, MSA-P, and DLB compared to controls, and the proportion of 8OHG-positive neurons was significantly greater in PD than in MSA-P or DLB. In addition, the ventral tier, the ventrolateral SN in particular, contained the highest proportion of 8OHG-positive neurons in PD patients. This subregion of the SN is the most

extensively damaged in PD.<sup>27</sup> Of note, 8OHG staining also was observed in areas outside of the SN including the nucleus raphe dorsalis, oculomotor nucleus, and some glial cells (Figure 1C). The extra-SN staining was moderate in the PD patients, weak in MSA-P and DLB, and absent in age-matched controls. Importantly, there was no significant staining in the cerebellum or cerebral cortex of PD patients. This pattern of immunoreactivity is identical to observations of others using a different immunohistochemical probe for oxidative damage in the midbrain of PD patients.<sup>5</sup> In combination, these results suggest that oxidative damage to nucleic acid is increased in midbrain structures of PD, MSA-P, and DLB patients compared to age-matched control individuals, is greatest in SN neurons compared to other midbrain structures, and mirrors the regional distribution of neurodegeneration both within the substantia nigra and in other brain regions.

One reason why SN neurons may experience more oxidative damage is that those neurons use dopamine as their major neurotransmitter.<sup>9,11-13</sup> Dopamine is metabolized enzymatically to produce  $H_2O_2$  and ultimately dihydroxyphenylacetate (dopac). Also, dopamine and related o-catechols are unstable molecules that can oxidize in the presence of transition metals to yield  $O_2^-$  and quinoid species.<sup>28</sup>  $O_2^-$ , a product of catechol autooxidation, can readily oxidize catechols thereby propagating ROS generation.<sup>29,30</sup> In addition,  $O_2^-$  can yield  $H_2O_2$  either spontaneously or enzymatically and then  $\cdot OH$  in the presence of transition metals such as iron. Furthermore, catechol metabolism may contribute indirectly to  $\cdot OH$  production by depleting cellular reduced thiols, a major defense mechanism against  $H_2O_2$ , via catechol thioether formation.<sup>8,31</sup>  $\cdot OH$  is the primary species that attacks nucleic acid to yield 8OHG. Indeed, intense oxidative DNA damage with 8OHG formation has been shown *in vitro* by catechols in the presence of transition metals.<sup>32</sup>

A major consequence of increased ROS production is inhibition of mitochondrial function, a phenomenon first observed in midbrain tissue from PD patients about 10 years ago.<sup>33</sup> In line with this observation, our results suggest that mitochondrial DNA is one of the primary targets of oxidative damage in PD patients, since 8OHG immunoreactivity was limited to the cytoplasm of neurons and the immunoreactivity was significantly diminished by DNase pretreatment. It is perhaps not surprising that mitochondrial DNA would accumulate substantially more oxidative damage than nuclear DNA, because mitochondrial DNA has no protective histone coat and its repair mechanisms are much less efficient than those of nuclear DNA.<sup>18</sup> Our study showed that 8OHG adducts were significantly more common in SN neurons of PD patients compared to age-matched controls as well as to MSA-P and DLB patients, even though there was a similar degree of neuronal loss in the SN of patients from all three groups. This important result indicates that 8OHG formation is not simply a consequence of the imminent cell death and suggests that mechanisms of oxidative damage to SN neurons in PD may be different from in MSA-P or DLB. It is possible that this greater 8OHG adduct accumulation in PD may contribute to the mitochondrial

dysfunction characteristic of this disease because many subunits of mitochondrial complex I are encoded by mitochondrial DNA.<sup>34</sup> Consistent with this hypothesis, others have been unable to demonstrate mitochondrial dysfunction in midbrain from MSA-P patients.<sup>35,36</sup>

In addition to mitochondrial DNA, our results suggest that the other major target of oxidative damage is cytoplasmic RNA. Like mitochondrial DNA, RNA is not covered by protective histones and does not have advanced repair mechanisms. Also, because RNA is single-stranded it is even more vulnerable to free radical-mediated damage. The consequences of free radical-mediated damage to RNA are not fully understood. It is conceivable that mRNA damage may result in abnormal protein translation. tRNA and rRNA damage could result in dysfunction of protein synthesis. In an experimental system, inactivation of ribosomes has been demonstrated when 28S rRNA is damaged by free radicals.<sup>37</sup> Little is known about the role of damaged RNA in neurodegenerative diseases. However, there is recent evidence that posttranscriptional modifications of RNA and protein synthesis may be altered in Alzheimer's disease<sup>38,39</sup> and that aberrant RNA is associated with decreased activity of a glutamate transporter in amyotrophic lateral sclerosis.<sup>40</sup> Certainly, more work is required to understand the potential role of oxidatively damaged RNA in the pathogenesis of neurodegeneration.

In summary, we observed increased frequency of 8OHG immunoreactive SN neurons in PD, MSA-P, and DLB patients compared to age-matched control individuals, with significantly more 8OHG immunoreactivity in PD than in MSA-P or DLB patients. 8OHG immunoreactivity was cytoplasmic and was present on both DNA and RNA. These results point to larger increases in oxidative damage to cytoplasmic nucleic acids in SN neurons from PD patients compared to patients with other nigral degenerative diseases and raise the possibility that oxidative damage to mitochondrial DNA and RNA may contribute to neurodegeneration.

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